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1. Blumenthal RD, et al. Cancer Res. 1992 Nov 1;52(21):6036-44.
2. Levin LV, et al. Cancer Immunol Immunother. 1987;24(3):202-6.

Thank you.

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Comparison of multiple anti-CEA immunotoxins active against human adenocarcinoma cells*

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Summary. Anti-carcinoembryonic antigen (CEA) immunotoxins constructed with multiple anti-CEA antibodies (goat and baboon polyclonal, and three murine monoclonal antibodies) by covalently linking them to the A chain of ricin via a disulfide bond all function as potent and specific toxins for CEA-bearing cells, suggesting that the CEA molecule is capable of directing productive internalization of ricin A chain. The high potency of anti-CEA immunotoxins apparently makes addition of ricin B chain unnecessary for high toxic efficiency, as in some other systems, because presence of the B chain reduces target cell specificity. Several characteristics of the immunotoxins which might account for their cytotoxic potency were studied. Equilibrium association constants of the goat, baboon, and murine monoclonal C-19 antibodies with fluid-phase CEA were determined by using Langmuir plots and were found to be 8.79, 6.61, and $8.13 \times 10^9 M^{-1}$, respectively, indicating the high and similar affinities of the three antibodies toward CEA. Radioimmunoassay binding studies of the three immunotoxins with ^{125}I-CEA showed that the antibody portions of the molecules retained the ability to form complexes with CEA after conjugation to ricin A chain. The maximum number of anti-CEA antibody molecules bound per cell, as demonstrated by ^{111}In-labeled C-19 binding assays with CEA-bearing cell lines, varied from 2.65×10^5 per cell for HT29 to 2.01×10^6 for LoVo, with an intermediate value of 1.17×10^6 per cell for WiDr. Cytotoxicity of the immunotoxins was assessed by inhibition of protein synthesis and expressed as a median inhibitory dose (ID_{50}). Comparison of the ID_{50}'s of each immunotoxin on the three cell lines has shown that the immunotoxin made of the monoclonal C-19 antibody is in general 6 to 7 times more cytotoxic than the goat and baboon antibody immunotoxins. The affinity of CEA-antibody binding is probably an important, but not a sole factor in determining the immunotoxin potency. The fact that the antibodies with very similar affinity toward fluid phase CEA make immunotoxins of different potency might indicate that interactions with membrane-bound CEA are more complex and/or the efficiency of internalization of various immunotoxins is different. An important factor in immunotoxin action appears to be the CEA content in target adenocarcinoma cells.

Introduction

Cytotoxic drugs used in modern cancer chemotherapy still lack desired specificity and may have devastating side effects on the healthy tissues in the body. Efforts have been made towards the development of agents with improved selectivity, including extensive research in the use of conjugates of cytotoxic agents with antitumor antibodies (immunotoxins) (for review, see [18]). Work in this laboratory on conjugates of ricin A chain, the intracellularly active enzymic portion of the plant toxin [15], with antibodies directed against carcinoembryonic antigen (CEA)-bearing colorectal tumor cells has shown promising results with selective in vitro toxicities 500 times higher than A chain alone [4, 9].

The development of any immunotoxin as an agent for clinical antitumor therapy will be facilitated by the availability of a method for predicting the potential usefulness of such immunotoxins against a particular tumor. Previous studies [12, 16, 19] have suggested several factors may account for killing efficiency of a target cell by an immunotoxin. They include the affinity of the antibody portion of the immunotoxin for the cell surface antigen, the number of cell surface antigen molecules per cell, and the efficiency with which bound immunotoxin is internalized into the cytosol to then inactivate ribosomes. In the present study, we determined the cytotoxic action of the five anti-CEA ricin A chain immunotoxins made from two polyclonal and three monoclonal antibodies. Three colorectal tumor cell lines with differring CEA content were used as target tissues.

Materials and methods

Antigen and antibodies. CEA was isolated from a human colonic carcinoma metastasis in the liver by the method of Newman et al. [13]. ^{125}I-Labeled CEA was purchased from Roche Diagnostics, Nutley, NJ, USA, as part of their commercial CEA assay kit. Murine monoclonal anti-human CEA antibody (C-19) was a kind gift from Dr. H. J. Hansen, Hoffman-LaRoche, Nutley, NJ, USA. Two murine monoclonal anti-CEA antibodies (anti-site 1 and anti-

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site 2) were obtained from Abbott Laboratories, Chicago, Ill., USA. Goat and baboon affinity purified anti-CEA antibodies have been isolated and characterized previously [5].

Cell lines. Human colorectal carcinoma cell lines producing CEA in culture (LoVo, WiDr, and HT29) were purchased from the American Type Culture Collection, Rockville, Md. USA.

Determination of association constants. Association constants for C-19 and goat and baboon polyclonal antibodies were determined using a modification of the Langmuir equation [1]. Formation of antigen-antibody complexes was determined by the radioimmunoassay (RIA) method described elsewhere [5]. For construction of the Langmuir graphs, each of the three antibodies was reacted with antigen at room temperature until equilibrium was reached (18 h). Then the antigen-antibody complexes formed were precipitated with 1 ml of zirconyl phosphate gel solution (Z-gel), pH 6.25 [6]. ^{125}I-CEA in the complexes was counted in a Packard Prias gamma counter programmed for automatic subtraction of background counts. For each antibody, the binding curve was performed. In the assay system, 1.25 ng of ^{125}I-CEA and increasing amounts of unlabeled CEA (0, 1.25, 2.50, 3.75, 5.00, or 7.50 ng) were reacted with a constant concentration of antibody in 3.5 ml of Roche-EDTA buffer.

The association constant for antigen-antibody interaction is K_a=[Ag-Ab complex]/[Ag] free × [Ab] free.

The Langmuir absorption isoterm $y/x = 1/k' \cdot 1/f + m$ is one method for determining the above antibody-antigen association constant [1]. In our experiments we used this equation with nanogram per milliliter units. We expressed the association constant (k') in milliliter per nanogram units; (y) was the total antibody concentration, (x) the bound antigen concentration, (f) the free antigen concentration, (m) was the nanograms of antibody which bound 1 nanogram of antigen, and (mx) was the bound antibody concentration.

By plotting both 1/f and y/x, obtained directly from the experiment, on abscissa and ordinate, respectively, then one can determine the slope 1/k' from the graph. Since $1/k' = (y/x - m) \times f$, then the dimension of antigen units cancels out. In order to convert k' into K_a, antibody concentration was converted into molar units. The molecular weight of each antibody was considered to be 150,000 daltons and its valency 2. Then the conversion coefficient is 7.5×10^{10}. In these experiments, slopes were determined by fitting the data points into a straight line by linear regression analysis (Table 1).

Synthesis of immunotoxins. The method is described in detail elsewhere [9]. Briefly, the following procedures were used. Ricin A chain was isolated from ricin (castor bean toxin) obtained from EY Labs. (San Mateo, Calif., USA). The preparation had a median inhibitory dose (ID_{50}) of 1.7×10^{-7}M for LoVo cells. Baboon and goat affinity purified antibodies and murine monoclonal antibodies at the 4 mg/ml concentration were dialyzed against two changes of buffer (0.05 *M* sodium bicarbonate/0.9% saline, pH 8.3) and then reacted with tenfold molar excess of the bifunctional coupling reagent N-succinimidyl 3-(2-pyridyldithio) propionate (SPDP) for 30 min at room temperature. The reaction products were dialyzed for 16 h at 4 °C with two changes of the above buffer. PDP-substituted antibodies were reacted with a fivefold excess of A chain overnight at room temperature. Reaction products were then separated by gel filtration on a Sephacryl S-300 column. The column yielded two peaks: a breakthrough peak consisting of high molecular weight material and a peak of unconjugated ricin A chain. The breakthrough fractions contained a mixture of species of antibody conjugated to one, two, three, or four ricin A chain molecules and unconjugated antibody. The proportion of conjugated antibody molecules gradually decreased with the increase of the distance of the fraction from the beginning of the peak. Fractions with the highest degree of substitution were detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of peak fractions in 7% gel.

Construction of binding curves for CEA and immunotoxins. Baboon anti-CEA antibody and ricin A chain immunotoxins made of C-19, baboon, and goat antibodies were diluted to 1:2000 of one OD_{280} unit in 0.01 *M* NaN_3 buffer, pH 6.75, containing 1 mg/ml of human serum albumin. Several volumes of each immunotoxin and baboon antibody (from 11 to 200 µl) were reacted with 1 ng of ^{125}I-CEA (100,000 cpm) in 5 ml of the total volume in Roche-EDTA buffer at room temperature overnight and the immunotoxin-CEA complexes formed were precipitated by Z-gel. The precipitates were counted in a gamma counter. The data generated were used to construct binding curves (see Fig. 1).

Inhibition of cellular protein synthesis. LoVo, HT29, and WiDr cells were dispersed with 0.25% trypsin and 0.02% EDTA from near-confluent T-flasks. The cells were suspended in leucine-free medium (minimum essential medium, 10% fetal calf serum (FCS), 1% antibiotics) and seeded into microtiter wells (7,000 cells in a final volume of 200 µl per well). The cells were incubated with media alone (control) or with specified additions of ricin A chain or each of the above immunotoxins for 46 h at 37° C in 5% CO_2. Thereafter, 0.1 µCi of ^{14}C-leucine (NEN, Boston, Mass., USA) was added to each well. Following a 3-h incubation the cells were collected onto glass fiber filters using a Mash II cell harvester. Incorporation of ^{14}C-leucine into cellular protein was measured by scintillation counting of the glass fiber discs.

Determination of total cellular CEA content in the cell cultures. Cells were plated at a concentration of 10^8 cells/flask, then grown for 10 days. They were lifted with 0.25% trypsin and 0.02% EDTA, washed once, counted, then dissolved in 6 *M* guanidine hydrochloride. CEA content was determined in a direct CEA RIA [5].

Cell binding assays for ^{111}In-labeled C-19. Near confluent cultures were dispersed with trypsin-EDTA, washed with phosphate-buffered saline (PBS), and an aliquot was counted in a counting chamber. Glass tubes (12×75 mm) were pretreated with 1% bovine serum albumin for at least 1 h and air dried. A volume of cell suspension equivalent to 6×10^5 cells (200–1000 µl) was added to each tube. Cells were pelleted by centrifugation at 2000/rpm (about 400 g) for 8 min and resuspended in 100 µl of ^{111}In-labeled C-19 antibody in PBS. The labeling of C-19 antibody with ^{111}In

Table 1. Determination of K_a by the Langmuir graph

	C-19	Baboon	Goat
Slope	9.22	11.34	8.53
$K_a \frac{1.5 \times 10^{11}}{2 \times \text{slope}} (M^{-1})$	8.13×10^9	6.61×10^9	8.79×10^9
Correlation coefficient	0.9936	0.9978	0.9913

The slope $1/k^1$ for each antibody was determined using the linear regression analysis fitting into the straight line. k^1 (expressed in ml/ng units) was converted into K_a (in M^{-1}) by considering the molecular weight of each antibody to be 150,000 daltons and its valency 2

was performed in accordance with the procedure of Hnatowich et al. [7]. The range of antibody concentrations was between 0.05 µg and 10 µg for each cell line. Each binding assay was done in triplicate, and controls consisted of a fourth tube containing labeled antibody and no cells. Cells were incubated for 2 h at 23° C with occasional mixing and then washed three times with cold PBS containing 2% FCS. If there was any delay between centrifugations, tubes were placed on ice. The absolute numbers of counts bound to cells were calculated by subtracting the counts in tubes containing label alone from counts in tubes containing label and cells.

Results

The association constants obtained for all three antibodies from the experiments with variable concentrations of CEA were very similar, indicating that the affinities of the three antibodies toward CEA are high (the order of 10^9 l/mole) and close to each other (Table 1). The association constants for anti-site 1 and anti-site 2 monoclonal antibodies to fluid-phase CEA were also of the order of 10^9 l/mole (unpublished data).

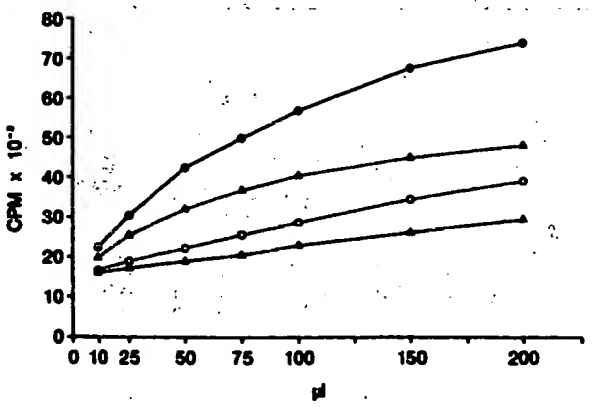

Fig. 1. Carcinoembryonic antigen (CEA) binding curves for the immunotoxins. Baboon anti-CEA antibody and three immunotoxins made of ricin A chain and C-19, baboon, and goat antibodies were diluted to 1:2000 of one OD_{280} unit in 0.01 *M* NaN_3 buffer, pH 6.75, containing 1 mg/ml of human serum albumin. then 11, 25, 50, 75, 100, 150, and 200 µl of each immunotoxin or baboon antibody were reacted with ^{125}I-CEA in the total volume of 5 ml of Roche-EDTA buffer, precipitated and counted. The binding curves were constructed by plotting microliters of the immunotoxin versus precipitated counts. ●———● baboon antibody, Δ-----Δ C-19 antibody-ricin A chain immunotoxin, O----O baboon antibody-ricin A chain immunotoxin, ▲——▲ goat antibody-ricin A chain immunotoxin

Table 2. Cytotoxicity of the immunotoxins for three colorectal adenocarcinoma cell lines

Cell line	CEA content (ng/10^7 cells)	ID_{50} (M) of immunotoxins			Ricin A chain
		C-19	Baboon	Goat	
HT29	0.065	3.8×10^{-8}	1.5×10^{-7}	$> 2.0 \times 10^{-7}$	7.5×10^{-7}
WiDr	0.125	4.1×10^{-8}	2.4×10^{-7}	$> 1.5 \times 10^{-7}$	3.0×10^{-7}
LoVo	10.500	3.1×10^{-10}	2.1×10^{-9}	7.7×10^{-9}	1.9×10^{-7}

The cells in microtiter wells (7×10^3 cells in 200 µl of leucine-free media with 10% of fetal bovine serum per well) were incubated with the addition of each of the above immunotoxins, or ricin A chain, or media (controls) for 46 h at 37° in 5% CO_2. Thereafter, the reagents were removed, cells washed and incubated with 0.1 µCi of ^{14}C-leucine per well. In 3 h the cells were collected in a cell harvester. ^{14}C-leucine incorporation was measured by scintillation counting of glass fiber discs

The maximum number of C-19 antibody molecules labeled with ^{111}In and bound by the cell lines employed in the study was 2.65×10^5 per cell for HT29, 1.17×10^6 per cell for WiDr, and 2.1×10^6 per cell for LoVo.

The next part of our work was to evaluate how efficient were immunotoxins made from these high affinity antibodies in inhibiting protein synthesis in the CEA-bearing tumor cells. Immunotoxin tumor cell killing is dependent on antibody delivering ricin A chain to the cell when the toxin inactivates ribosomes [19]. It is important that the antigen specificity of the antibody molecule after toxin conjugation be unimpaired. RIA binding studies on these preparations showed that all the immunotoxins formed complexes with ^{125}I-CEA indicating that the antibody portion of the molecule was functional (Fig. 1), though to a lesser degree than in unconjugated baboon antibody. This can be explained by the fact that conjugated ricin A chain(s) might affect the antigen-combining site of the antibody by creating steric hindrance. Indirect immunofluorescent staining with C-19-ricin A chain immunotoxin was performed and showed specific cellular binding, again indicating functional antibody [10].

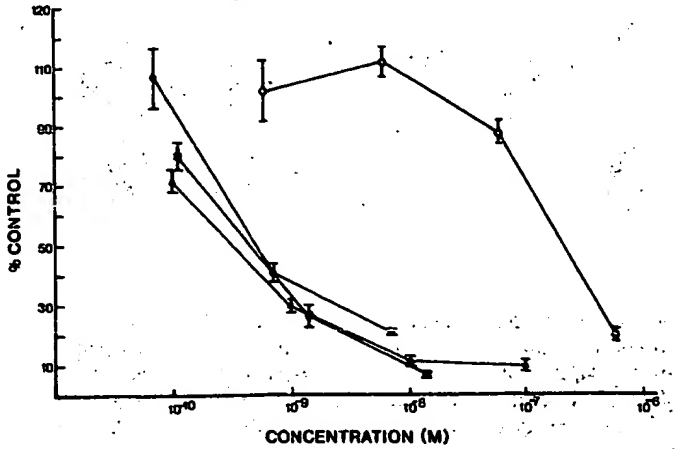

Fig. 2. Inhibition of ^{14}C-leucine incorporation in LoVo cells by specific immunotoxins and ricin A chain. O———O ricin A chain. ●———● anti-site 2 antibody – ricin A chain immunotoxin, ■———■ anti-site 1 antibody – ricin A chain immunotoxin, ▲———▲ C-19 antibody – ricin A chain immunotoxin. The conditions of the experiment were the same as in the legend to Table 2.

Cytotoxicity was assessed by inhibition of protein synthesis in the cells after a 46-h incubation with immunotoxin and subsequent pulse with ^{14}C-leucine, and expressed as ID_{50}. The results of these experiments are shown in Table 2. These data indicate that all three anti-CEA antibody-ricin A chain preparations mediated selective A chain toxicity toward CEA-bearing cells. For example, in LoVo cells these immunotoxins differed in potency of inhibition of ^{14}C-leucine incorporation over A chain alone from about 25 (goat) to 90 (baboon) to 600 times (C-19). The sensitivity of human adenocarcinoma cell lines to each of the immunotoxins is different.

In addition to the above-mentioned immunotoxins, two immunotoxins made from anti-site 1 and anti-site 2 anti-CEA monoclonal antibodies, in a 46-h protein synthesis inhibition assay in LoVo cells, showed ID_{50}'s of about 5×10^{-10} *M* (Fig. 2).

Discussion

The present work has shown that anti-CEA immunotoxins constructed with multiple anti-CEA antibodies (goat and baboon polyclonal and three murine monoclonal antibodies) all function as potent and specific anti-CEA positive cell cytotoxins.

Previous studies in other systems [12, 16, 19] have suggested that several factors could account for the immunotoxins cellular toxicity. These factors include the affinity of the antibody portion of the immunotoxin for the cell surface antigen, the number of cell surface antigen molecules per cell, and the efficiency with which the toxic moiety gains access to the cytosol to then inactivate ribosomes.

In our system, the most important factor for cytotoxicity appeared to be the concentration of CEA on the cell membrane. Total cellular CEA content, determined by RIA (see Table 2) was assumed to reflect the membrane CEA density [2]. In LoVo cells it was about 80 and 150 times higher than in WiDr and HT29 cells, respectively. Direct binding assays with ^{111}In-labeled C-19 antibody have shown that LoVo cells had the highest, and HT29 cells the lowest membrane CEA expression, with an intermediate value for WiDr. The ID_{50} of each immunotoxin with LoVo cells was approximately 100 times lower than the two other cell lines.

Comparison of the ID_{50}'s of each immunotoxin on the three cell lines showed that the immunotoxin made of the monoclonal C-19 antibody was in general 6 to 7 times more cytotoxic than the goat and baboon antibody immunotoxins. We do not know the exact ricin A chain content in the three preparations, however, by comparison of gel electophoresis patterns of the immunotoxins (data not shown) it appears that the preparations were similar in ricin A chain content.

All three antibodies used for construction of the immunotoxins had very similar equilibrium association constants with fluid-phase CEA characterizing their affinities (Fig. 1, Table 1). After the ricin A chain conjugation antibodies appeared to have similar binding characteristics to the respective native antibodies. The differences in the cytotoxicities of the immunotoxins may be related to the CEA determinants being recognized. Both the goat and baboon antibodies recognize CEA-specific site determinants while C-19 is against a common site determinant. Antibody binding to a common site determinant on CEA may occur more easily at the cell surface than binding to a specific site. Preliminary data supporting that concept has been given elsewhere [8] Another possible explanation which does not preclude the first one, might be that the reaction of antibodies or immunotoxins with the membrane surface bound antigen may be quite different from fluid-phase antigen-antibody reaction. The differences observed between the three anti-CEA antibody-ricin A chain immunotoxins may be explained by differences in cell surface phenomena.

It was found in binding experiments with ^{125}I-labeled mouse monoclonal antibodies to three cell surface antigens of rat thymocytes that the theories based on equilibrium binding are inappropriate, because the results of the binding could be interpreted on the basis of irreversible reactions [11]. To evaluate binding to the cell, the association rate constant (k_{+1}) and dissociation rate constant (k_{-1}) seem to be most important. In addition, many researchers believe now that the carrier antibody of immunotoxin should not only deliver A chain to the target cell, but also be internalized, though it has been shown for only two antibodies used for the construction of immunotoxins [3, 16]. Our flow cytometry studies of C-19 antibody on LoVo cells suggest that after binding to the cell surface the antigen-antibody complexes undergo modulation from the cell surface by endocytosis [10, 14]. The results with radiolabeled goat antibody from another laboratory indicate a similar process in CEA-bearing HCT-8R cells [17]. Thus, the affinity is probably not directly related to cytotoxicity, because the equilibrium on the cells is hardly achieved. The difference in cytotoxicity of the immunotoxins constructed of monoclonal and polyclonal antibodies observed in this work, could probably be due to different efficiencies of the toxin uptake by the cell. If modulation is responsible for the toxin uptake in our system, then the results imply that the rate of modulation should be faster with monoclonal than with polyclonal antibodies. We can speculate that either monoclonal antibodies have higher k_{+1}, or the orientation of CEA on the cell surface (or availability of a specific epitope) is more favorable for binding monoclonal than two other antibodies.

Anti-CEA immunotoxins have high, though lower than native ricin potency toward CEA positive cells, while they have little intrinsic toxicity toward CEA negative control cells [4, 9]. Other antigen systems apparently have required the addition of ricin B chain for high potency of the cytotoxic effect, however, target cell specificity was reduced [19]. This gives a hope that the efficiency of ricin A chain delivery by anti-CEA immunotoxins is sufficient for an active in vivo immunotoxin for CEA-bearing cells. Probably making a "cocktail" of immunotoxins constructed with monoclonal antibodies each specific for a different epitope on the CEA molecule would be even a more efficient way of achieving maximal target cell killing.

The present work has led us to the conclusion that the affinity of the antigen-antibody binding is not the major factor in immunotoxin action on the cell, since antibodies with similar affinity to CEA and with similar ricin A chain content acted as immunotoxins with different potency. The membrane CEA density appears to be a more important factor in immunotoxin action. The association and dissociation rate constants for membrane-associated CEA with the antibody-ricin A chain immunotoxins remain to

be elucidated. This work does indicate that CEA-directed immunotoxins may be potential antitumor reagents.

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